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Regenerative Neurogenesis from Neural Progenitor Cells Requires Injury-Induced Expression of Gata3

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SUMMARY

The adult zebrafish brain, unlike mammalian counterparts, can regenerate after injury owing to the neurogenic capacity of stem cells with radial glial character. We hypothesized that injury-induced regenerative programs might be turned on after injury in zebrafish brain and enable regenerative neurogenesis. Here we identify one such gene—the transcription factor *gata3*—which is expressed only after injury in different zebrafish organs. Gata3 is required for reactive proliferation of radial glia cells, subsequent regenerative neurogenesis, and migration of the newborn neurons. We found that these regeneration-specific roles of Gata3 are dependent on the injury because Gata3 overexpression in the unlesioned adult zebrafish brain is not sufficient to induce neurogenesis. Thus, *gata3* acts as a specific injury-induced proregenerative factor that is essential for the regenerative capacity in vertebrates.

INTRODUCTION

In mammalian brains, the constitutive production of new neurons is limited to two subregions of the telencephalon and relies on the presence of stem cells with glial character (Alvarez-Buylla et al., 2002; Morrens et al., 2012). After injury, glial cells may proliferate but form neurons only poorly and hence cannot accomplish sufficient regenerative neurogenesis in most vertebrates (Arvidsson et al., 2002; Yiu and He, 2006; Rolls et al., 2009; Kempermann, 2010; Robel et al., 2011). In contrast, adult zebrafish have a remarkable ability to regenerate the central nervous system (Becker and Becker, 2008; Antos and Brand, 2010; Poss, 2010; Kizil et al., 2012a). Besides widespread adult neurogenic activity (Grandel et al., 2006; Adolf et al., 2006; Chapouton et al., 2007; Kaslin et al., 2008; Ganz et al., 2010; Kizil et al., 2012a), zebrafish brains can also regenerate severe traumatic brain injuries, during which glial progenitors are stimulated to divide and replenish lost neurons (Kroehne et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012). However, the molecular mechanisms involved in the initiation and maintenance of the injury-induced regenerative response in the adult zebrafish brain are unknown. Conceivably, the profound differences in the regenerative capacity between the brains of zebrafish and mammals (Dinsmore, 1991; Kempermann, 2010; Poss, 2010) might be linked to these mechanisms. Thus, a comparison between the regenerative mechanisms in mammals and zebrafish could reveal genes underlying the differential responses, which could then be harnessed for therapeutic applications in human neurological disorders and acute injuries. Here, we sought to identify injury-induced genes in the adult zebrafish brain using a traumatic brain injury paradigm to the telencephalon that we established previously (Kroehne et al., 2011). We find that the transcription factor *gata3* is specifically associated with the regenerative state of brain and several other zebrafish tissues and that *gata3* is required for telencephalon and fin regeneration. Overall, we demonstrate that Gata3 is essential to trigger a molecular program in radial glia cells after injury, thus enabling the regenerative neurogenesis from these progenitor cells.

RESULTS AND DISCUSSION

Stab-Lesion Injury Induces *gata3* Expression in the Adult Zebrafish Telencephalon

To identify regeneration-associated factors in the adult zebrafish brain, we compared the transciptomes of uninjured and injured telencephalons, coupled to a random-pick secondary in situ hybridization (ISH) screen on cross-sections (C.K. and M.B., unpublished data; Figure 1A). The zinc-finger transcription factor *gata3* is not expressed in embryonic (based on quantitative real-time PCR and ISH; data not shown) and adult zebrafish telencephalon (Figure 1B) but was induced in the telencephalon after injury (Figure 1C). Immunohistochemistry (IHC) confirmed the injury-dependent induction of Gata3 (Figures 1D and 1E) along the entire ventricular region (Figures 1E1–1E4), which contains the radial glia cells (RGCs) as neurogenic progenitors (Kroehne et al., 2011; Rothenaigner et al., 2011). Gata3 is induced in proliferating and nonproliferating RGCs (marked by her4.1:GFP) following the injury (Figures 1F and 1G; Figure S1A available online). *gata3* expression is first observed at 12 hr after the lesion (hpl) in ventricular cells that are mainly nonproliferative (Figures 1G, S1B, and S1B′). The number of *gata3*-positive RGCs

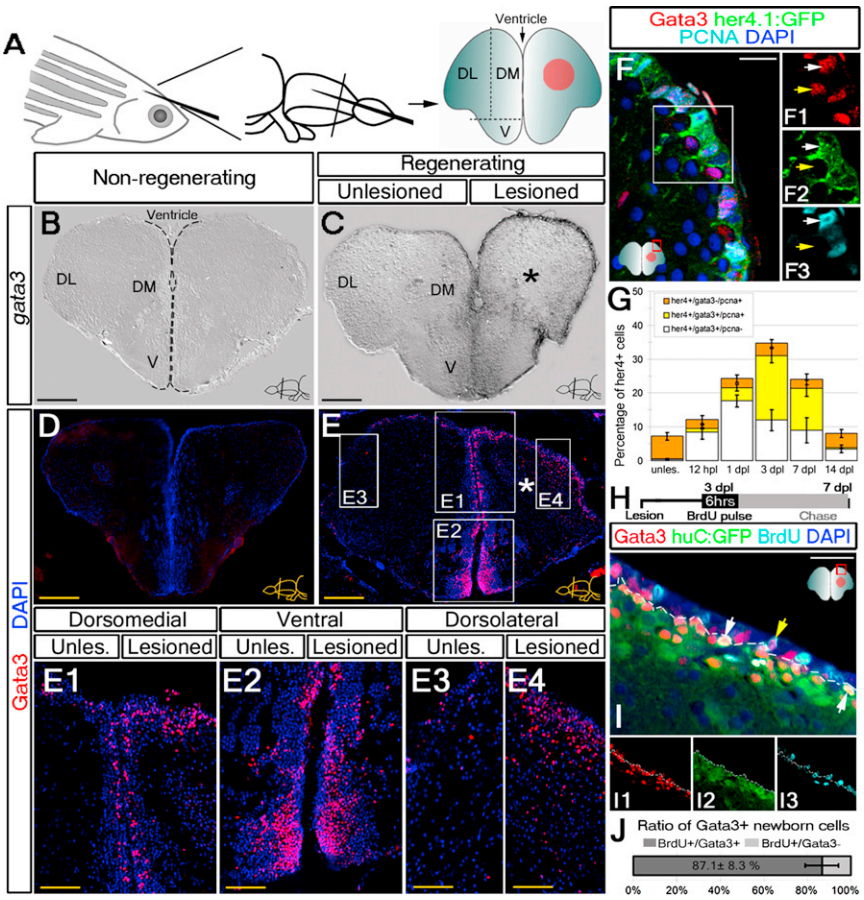

Figure 1. Gata3 Expression in Radial Glia and Newborn Neurons Is Injury Induced

(A) Injury paradigm in adult zebrafish telencephalon. DM, dorsomedial; DL, dorsolateral; V, ventral. Red circle is the injury site.

(B) *gata3* in situ hybridization (ISH) in uninjured adult telencephalon.

(C) *gata3* expression in the lesioned hemisphere after the stab lesion (*) at 3 dpl.

(D) Gata3 immunohistochemistry (IHC) in unlesioned telencephalon.

(E) Gata3 IHC on lesioned telencephalons. (E1–E4) High-magnification images from different regions.

(F) Gata3-positive cells in the ventricular region colocalize with her4.1:GFP (white arrows). Gata3 is also present in GFP-negative cells in close proximity to the GFP-positive ventricular region (yellow arrows). (F1–F3) Single fluorescence channel of the frame in (F).

(G) Time course quantification graph for *her4*-positive radial glial cells (RGC) in regard to *gata3* expression and proliferation. Nonproliferating *gata3*-positive RGCs, white; proliferating *gata3*-positive RGCs, yellow; proliferating RGCs that do not express *gata3*, orange.

(H) Experimental scheme for BrdU treatments.

(I) Gata3, huC:GFP, BrdU triple IHC. Triple-positive cells, white arrows. BrdU-Gata3 double-positive cells lacking huC:GFP in the ventricular region, yellow arrows.

(J) Graph for the ratios for BrdU-Gata3 double-positive and only BrdU-positive cells. Dashed lines indicate the boundary between huC:GFP-negative and positive zones (I–I3).

Scale bars: 100 μm (B–E); 50 μm (E1–E4); 20 μm (F and I). n = 8 fish for (B–E4); 4 for (F), 4 for (G), and 5 for (H and J). Data are represented as mean ± SEM. See also Figure S1.

increases until 3 dpl and then declines to unlesioned levels at 14 dpl (Figures 1G and S1C–S1G). Gata3 is mainly observed in her4.1-positive RGCs, and the maximum proportion of *gata3*-positive proliferating RGCs (her4+/Gata3+/PCNA$^+$) is observed at 3 dpl (54.6% ± 6.1% of all Gata3-positive cells; Figure 1G). Interestingly, there is also a relatively constant level of proliferating radial glial cells that are not expressing *gata3* (her4+/Gata3−/PCNA$^+$; orange section in Figure 1G), suggesting that *gata3* expression might demarcate the radial glial cells that are induced after the lesion but not the constitutively proliferating progenitors.

Gata3-positive cells were also detectable outside the ventricular zone at 3 dpl (Figure 1F), and these cells might be newborn neurons. To address this, we used a transgenic zebrafish line that marks postmitotic neurons, *Tg*(*huC:GFP*) (Figures S1H–SI3) and a short Bromodeoxyuridine (BrdU) pulse-chase experiment (Figure 1H). We found that Gata3 was expressed in the majority (85.7% ± 6.2%) of the newborn neurons following injury (Figures 1I–1J). At 7 dpl, the majority of the Gata3-positive cells are newborn neurons (79.4% ± 4.7%). We also identified that at 7 dpl, HuC/D-positive neuroblasts that had already been postmitotic at the time of the lesion also express Gata3 de novo (Figures S1J and S1K). Yet, this cell population (BrdU−, Gata3+, HuC/D+) constitutes only a small fraction of all Gata3-positive cells at 3 dpl (4.6% ± 2.1%; yellow arrows; Figures S1J and S1K).

Inflammation is an immediate response to the injury in the adult zebrafish brain (Kroehne et al., 2011), and Gata3 was previously shown to be expressed in immune cells (Ho et al., 2009). Therefore, we analyzed whether Gata3 is also expressed in leukocytes using L-plastin as a marker (Figures S1L–S1O). L-plastin-positive cells constituted only a minor fraction (5.1% ± 0.8%) of all Gata3-positive cells (Figure S1P). Collectively, our studies on Gata3 expression in different cell types indicated that Gata3 is induced in nonproliferating RGCs within the first day after lesion, becomes predominant in the proliferating RGCs at 3 dpl, and persists in the daughter newborn neurons at 7 dpl. Gata3-positive cells can no longer be detected at 14 dpl (Figure S1), suggesting a transient early role for this factor during adult zebrafish brain regeneration. Gata3 is also expressed in L-plastin-positive macrophages/microglia and neuroblasts that do not proliferate after the lesion, yet these cell populations constitute less than 10% of the whole Gata3-expressing cells at any time point after the lesion. We also observed that Gata3 is the only Gata-factor expressed in the telencephalon after lesion (Figures S1Q and S1R), suggesting a specific relevance of Gata3 to the regenerative response.

Because various types of neurons are generated from the RGCs in the adult dorsal zebrafish telencephalon (Grandel et al., 2006; Kroehne et al., 2011; Rothenaigner et al., 2011), we examined the neuronal subtypes that expressed Gata3. At

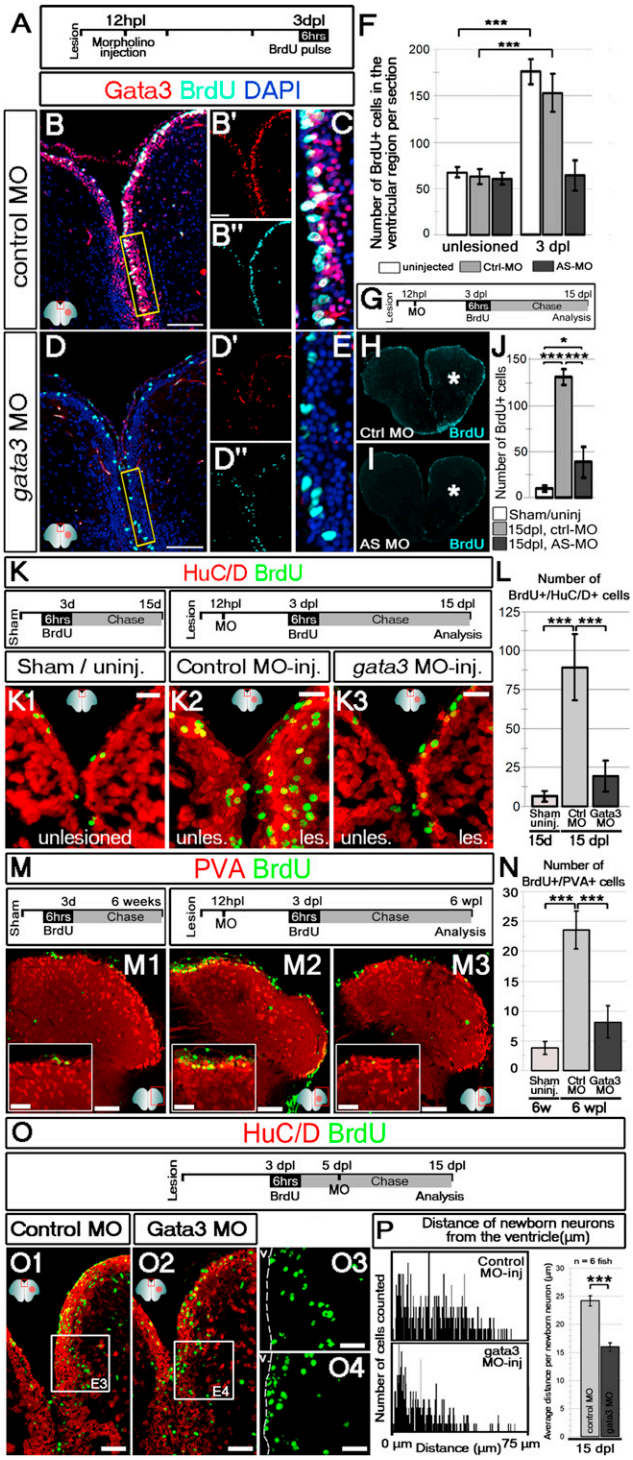

Figure 2. Gata3 Is Required for Reactive Cell Proliferation, Reactive Neurogenesis, and Tissue Distribution of Newborn Neurons

(A) Morpholino injection paradigm.

(B) Gata3 and BrdU IHC for control morpholino-injected brains.

(C) High magnification of the framed region in (B).

(D) Gata3 and BrdU IHC for Gata3-antisense morpholino-injected brains.

(E) High magnification of the framed region in (D).

(F) Quantification graph for BrdU-positive cells in the ventricular region of unlesioned and 3 dpl telencephalic hemispheres.

(G) BrdU pulse-chase experiment.

(H) BrdU IHC on control morpholino-injected brains.

(I) BrdU IHC on Gata3 antisense morpholino-injected brains.

(J) Quantification graph. Scale bars, 50 μm. n = 7 fish for (B–F) and 4 for (G–J). Data are represented as mean ± SEM.

(K) Morpholino-injection and BrdU pulse-chase schemes for reactive neurogenesis assay. (K1–K3) HuC/D-BrdU double IHC on sham/uninjected, control morpholino-injected, and *gata3* morpholino-injected telencephalons.

(L) Quantification graph.

(M) Morpholino-injection and BrdU pulse-chase schemes for reactive neurogenesis assay for PVA neuronal subtype. (M1–M3) PVA-BrdU double IHC on sham/uninjected, control morpholino-injected, and *gata3* morpholino-injected telencephalons.

(N) Quantification graph.

(O) Morpholino-injection and BrdU pulse-chase schemes to assay for migration of newborn neurons. (O1 and O2) HuC/D-BrdU double IHC on control morpholino-injected and *gata3* morpholino-injected telencephalons. (O3 and O4) High-magnification images of BrdU staining in framed regions of (E1 and E2). Dashed line is the ventricular surface.

(P) Histogram and bar graph depicting the relative distance distribution of HuC/D-BrdU positive newborn neurons from the ventricle and the average distance migrated by a newborn neuron in control- and antisense morpholino-injected telencephalons. Scale bars: 20 μm (K1–K3, insets of M1–M3, O3, and O4) and 50 μm (M1–M3, O1, and O2). n = 8 fish for (K–L), 6 for (M–N), and 4 for (O–P). Data are represented as mean ± SEM.

See also Figures S2 and S3, and Tables S1 and S2.

7 days postlesion (dpl), newborn Gata3-positive cells expressed various neuronal or glial markers (Figures S1S–S1X), suggesting that *gata3* partakes in regeneration of most or all neuronal and nonneuronal subtypes in the telencephalon.

Gata3 Is Required for Reactive Proliferation of the Progenitors, Reactive Neurogenesis, and Migration of Newborn Neurons

Expression of Gata3 in the adult zebrafish telencephalon after injury was observed also in proliferating progenitors (Figures 1F, 1I, and S2A–S2D″). We thus hypothesized that Gata3 might be required for injury-induced proliferation of radial glial cells (RGCs). To test this, we knocked down Gata3 after the injury in periventricular cells by cerebroventricular microinjection (CVMI) of functional *gata3* morpholinos (Figures S2E–S2M), a method that we recently developed to allow knockdown of gene function in ventricular zone cells (Kizil and Brand, 2011). After CVMI, we treated the fish with BrdU at 3 dpl and analyzed the BrdU and Gata3 immunoreactivity in the unlesioned control and 3 dpl experimental brains (Figure 2A). We found that at 3 dpl, Gata3 and BrdU-positive cells are present throughout the ventricular regions in the control morpholino-injected (ctrl-MO) telencephalons (Figures 2B and 2C), whereas *gata3*-morphants (AS-MO) lack Gata3 protein along the ventricle (Figures 2D and 2E). Interestingly, knocking down *gata3* reduces the number of injury-stimulated proliferation at 3 dpl significantly, to the level characteristic for unlesioned telencephalon (Figure 2F). PCNA immunostainings confirmed these results further, showing that knockdown of Gata3 production prevented injury-induced proliferation after injury in the adult zebrafish telencephalon (Figures S2N–S2O).

Injury-induced (reactive) proliferation in lesioned adult zebrafish telencephalon leads to production of neurons from RGCs (Kroehne et al., 2011). Because knocking down Gata3 strongly reduced reactive cell proliferation of the RGCs, we examined

whether this also reduces the injury-stimulated production of new cells. We observed that Gata3 knockdown significantly reduces the number of BrdU-labeled cells at 15 dpl (72.2% ± 6.4%) (Figures 2G–2J). To test whether production of newborn neurons (reactive neurogenesis) is also reduced, we performed double immunostaining for the early neuronal marker HuC/D and BrdU at 15 dpl after BrdU administration (Figures 2K–2K3). We observed that upon Gata3 knockdown, the number of newborn neurons is significantly reduced (80.1% ± 9.5%) to the level characteristic for uninjured brains (Figure 2L).

The pronounced reactive cell proliferation of the RGCs after a lesion was shown to peak at 3 days postlesion (dpl) and to persist in a gradually decreasing manner until 14 dpl, when the ventricular cell proliferation returns to constitutive levels (Kroehne et al., 2011). Even if the initial burst of reactive proliferation of the RGCs is blocked by Gata3 knockdown, the CVMI knockdown is likely to be transient, estimated to be most effective until about 5 days postinjection (Kizil and Brand, 2011). Thus, the remaining constitutive neurogenesis could conceivably compensate for the lack of regenerative neurogenesis. We therefore asked if neurons can be regenerated in the long-term by any other secondary mechanism in the brains, where injury-induced reactive proliferation was reduced by knocking down *gata3*. We analyzed the number of newborn Parvalbumin (PVA+/BrdU+) neurons of the dorsal telencephalon at 6 weeks postlesion (Figures 2M–2M3). We found that compared to sham controls, injured telencephalon shows enhanced production of PVA neurons but that knocking down *gata3* still reduces production of newly born PVA neurons significantly (by 67.1% ± 10.1%) (Figure 2N). These results indicate that the initial burst of injury-induced proliferation of the RGCs is indeed critical for successful regenerative neurogenesis of the adult zebrafish telencephalon and cannot be efficiently compensated by ongoing or later constitutive neurogenesis within the tested time period.

Following injury, Gata3 is also expressed in maturing neurons within the periventricular zone of the telencephalon (yellow arrows marking the GFP-negative cells in close proximity to the GFP-positive RGCs at the ventricular region in Figure 1F). This suggests that Gata3 might have other roles in addition to controlling proliferation after brain injury. Therefore, we investigated possible functions of *gata3* in survival and/or migration during neuronal differentiation. TUNEL stainings at 1 and 3 dpl for control and *gata3* morpholino-injected brains showed no significant difference in the number of apoptotic cells at these time points (Figures S2P–S2S). These results indicate that *gata3* is not required for cell survival. In order to analyze the distribution of newborn neurons relative to their place of birth in the ventricular zone, we treated the lesioned brains with BrdU at 3 dpl, injected the control and Gata3 morpholinos at 5 dpl, and counted the BrdU-positive neurons at 15 dpl (Figures 2O–2O4). This experiment would label the reactively proliferating RGCs at 3 dpl, and until the morpholino injection at 5 dpl, some of the proliferating RGCs would become postmitotic and differentiate into neurons that would start migrating toward the lesion site, as described before (Kroehne et al., 2011). The Gata3 knockdown at 5 dpl would then block further the production of neurons and allow visualization of how far the postmitotic neurons have migrated. As a result, we found that compared to the control morphant brains, knocking down Gata3 led to significantly altered tissue distribution and average migration distance of the newborn neurons (Figure 2P). We also observed that the total number of BrdU-positive neurons did not change upon Gata3 knockdown in comparison to the control morpholino injection (data not shown), confirming our previous results that *gata3* is not required for survival. In summary, our results suggest that *gata3* is required for neuronal regeneration of the lesioned adult zebrafish telencephalon, specifically for injury-induced proliferation of radial glial cells (RGCs), reactive neurogenesis, and proper distribution of newborn neurons.

By using a modified version of the CVMI method (Figures S3A–S3G) and transfecting the ventricular cells of the telencephalon with plasmids expressing Gata3 and GFP (Figures S3H–S3O), we overexpressed Gata3 and found that Gata3 alone is not sufficient to increase the ventricular cell proliferation and regenerative neurogenesis in unlesioned brains. This suggests that Gata3 requires the injury context and acts synergistically with those cues. Additionally, we found that increased Gata3 expression after a lesion does not further increase the proliferation levels at the ventricle (Figures S3P–S3R), suggesting that additional cells cannot be recruited and/or that the proliferation response is at its maximum level and cannot be enhanced further.

gata3 Expression Is Induced in Regenerating Zebrafish Organs after Injury

To investigate whether *gata3* might be involved in regeneration of other adult organs outside the central nervous system, we performed ISH on control and amputated fish heart and caudal fin (Figure 3). In contrast to sham-operated hearts (Figures 3A–3A′), *gata3* is expressed in hearts at 3 days postamputation (dpa) (Figure 3B–3B′). Sense probe control indicates the specificity of the in situ staining (Figures 3B′1–B′2). Similarly, whereas *gata3* expression levels are undetectable in the control fins (Figure 3C), fin amputation induces *gata3* expression based on ISH (Figures 3D–3G) and quantitative real-time PCR analyses (Figure 3H). The induction of *gata3* expression can first be seen at 1 dpa during the wound healing phase (Figure 3D). As regeneration proceeds in the caudal fin, *gata3* expression is observed in the regenerating portion of the fin and overlapping to the blastema and the interray epithelium (Figures 3E and 3F). At 7 dpa when the proximal structures of the fin regenerates, *gata3* expression levels reduce significantly (Figure 3G), suggesting a transient early role of *gata3* in the caudal fin regeneration. When we used a morpholino-knockdown-based regenerative outgrowth assay (Figure 3I; Kizil et al., 2009), we found that *gata3* knockdown significantly reduced regenerative outgrowth of the fin by 58.3% ± 8.9% (Figures 3I′ and 3J), indicating that *gata3* expression is necessary for regeneration of the caudal fin. We observed that *gata3* knockdown reduces cell proliferation in the blastema and the interray tissue significantly by 64.2% ± 14.3% (Figures 3K–3M), comparable to the reduction in the extent of regenerative outgrowth (Figure 3J). In control antibody stainings for Gata3, we observed that antisense morpholinos efficiently reduce the specific Gata3 signal in the epithelial and interray tissue and reduce the regenerative outgrowth of the fin (Figures 3N–3O′). We also found that *gata3* expression was induced after injury in other adult zebrafish tissues, such as cerebellum, optic tectum, spinal cord, and liver (data not shown).

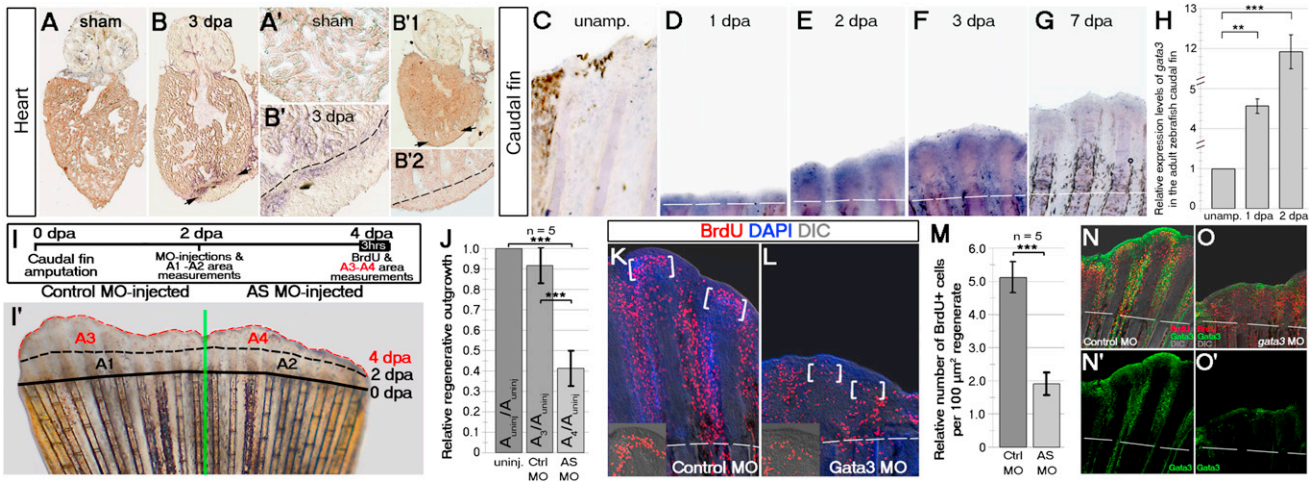

Figure 3. Gata3 Expression Is Injury Induced in Regenerating Heart and Caudal Fin and Is Required for Caudal Fin Regeneration

(A) *gata3* ISH on sham-operated zebrafish heart. (A′) High-magnification image.

(B) *gata3* ISH on amputated heart. Amputation plane, black arrows. (B′) High-magnification image. Dashed lines, amputation plane. (B′1) *gata3* ISH with the sense probe control on amputated heart. (B′2) High-magnification image from the amputation margin of the heart in (B′1).

(C–G) Time course *gata3* ISH on adult zebrafish caudal fin: unamputated (C), 1 dpa (D), 2 dpa (E), 3 dpa (F), and 7 dpa (G).

(H) Quantitative real-time PCR analyses for *gata3* expression on crude lysates of the unamputated, 1 dpa, and 3 dpa caudal fins. Bars represent ± SEM.

(I) Experimental scheme for caudal fin outgrowth assay. (I′) A 4 dpa caudal fin. One lobe is injected with control morpholino (Ctrl-MO) and the other with *gata3* antisense morpholino (AS-MO) at 2 dpa. A1, Area regenerated between 0–2 dpa in the control lobe before injection. A2, Area regenerated between 0–2 dpa in the AS-MO lobe before injection. A3, Area regenerated between 2–4 dpa after Ctrl-MO injection. A4, Area regenerated between 2–4 dpa after AS-MO injection.

(J) Quantification graph for the relative regenerative outgrowth between 2 and 4 dpa compared to uninjected regenerates. A_{uninj}, Area regenerated between 2–4 dpa without injection.

(K) BrdU IHC on control MO-injected lobe. Parenthesis indicate the blastema. Inset is a high magnification for the blastema of one fin ray. Bars represent ± SEM.

(L) BrdU IHC on *gata3* MO-injected lobe. Parenthesis indicate the blastema. Inset is a high magnification for the blastema of one fin ray.

(M) Quantification graph for the relative number of BrdU-positive cells in ctrl-MO and AS-MO-injected fins. Scale bars 100 μm; data are represented as mean ± SEM.

(N) BrdU-Gata3 double immunostaining shown on DIC image of the fin in (K). (N′) Gata3 staining alone is shown.

(O) BrdU-Gata3 double immunostaining shown on DIC image of the fin in (L). (O′) Gata3 staining alone is shown. n = 3 fish for (A–B′), 4 for every set for (C–G), 3 for (H), 6 for (I–J), 4 for (K–M), and 4 for (N–O′).

Overall, these results indicate that the injury-dependent activation of and requirement for *gata3* is not limited to the central nervous system but also prevails in other zebrafish organs, suggesting a general involvement of *gata3* in regeneration programs of zebrafish tissues.

Fgf Signaling Is Required for *gata3* Expression after the Injury

The Fgf signaling pathway was previously shown to be an integral regulator of the regeneration of heart and caudal fin tissue (Lepilina et al., 2006; Wills et al., 2008). In adult zebrafish brain, progenitor cells of the adult cerebellum and telencephalon also require Fgf signaling for proliferation and maintenance (Kaslin et al., 2009; Ganz et al., 2010). Therefore, we examined whether Fgf signaling is required to induce *gata3* expression after the injury, by using heterozygous heat-shock-inducible dominant negative Fgfr1-EGFP transgenic fish (Lee et al., 2005) (Figure 4A). Compared to control (nontransgenic, lesioned, and heat-shocked) brains (Figure 4B), transgenic animals (lesioned and heat shocked) showed a dramatic reduction in the number of Gata3-positive cells in the ventricular region after injury (Figure 4C). In the medial region the reduction was pronounced, and no Gata3-positive cells remained in the ventricular region after blocking Fgf signaling (Figures 4D–4E). ISH for *gata3* indicated that the reduction in the Gata3 protein levels upon Fgf-blockage is due to abolished *gata3* gene expression (Figures 4F–4G). Using the same transgenic line, we also observed that *gata3* expression in the regenerating caudal fin is dependent on Fgf signaling (Figures 4H–4H″). Blocking Fgf signaling during the whole regeneration process until 3 dpa blocked *gata3* expression (Figures 4H–4H″). Similarly, a short pulse of heat shock leads to abolishment of *gata3* expression as detected by Gata3 immunohistochemistry (Figures 4I–4I″). These results suggest that Fgf signaling is required for injury-induced *gata3* expression in regenerating zebrafish tissues and that Fgf signaling regulates Gata3 expression. In order to address whether or not the epistatic interaction between the Fgf signaling and *gata3* expression was an injury-specific phenomenon, we analyzed the expression of *gata3* in zebrafish embryos upon blocking Fgf signaling (Figures 4J–4M′). We found that upon blocking Fgf signaling, the expression of Fgf-responsive genes (such as *spry4*) was lost in zebrafish embryos (Figures 4J–4K′), whereas *gata3* expression was not affected (Figures 4L–4M′). These results indicate that Fgf signaling regulates *gata3* expression in an injury-dependent context.

We found that continuous blockage of Fgf signaling from shortly before the injury until 3 dpl abolished *gata3* expression in the adult zebrafish telencephalon (Figure 4); however, this

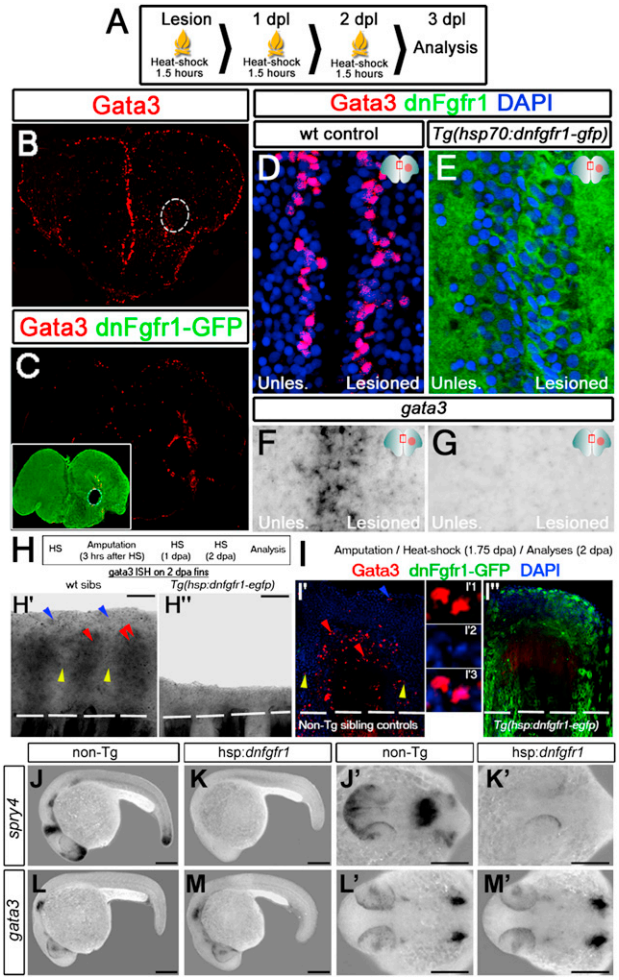

Figure 4. Fgf Signaling Is Required for Injury-Induced *Gata3* Expression

(A) Heat-shock scheme.

(B) Gata3 IHC on 3 dpl heat-shocked sham control fish telencephalon.

(C) Gata3 IHC on 3 dpl heat-shocked Tg(*hsp:dnfgfr1-gfp*) fish telencephalon. Inset: double IHC for GFP and Gata3, showing uniform dnFgfr1-GFP.

(D) High magnification from dorsomedial region of (B) with DAPI.

(E) High magnification from dorsomedial region of (C) with DAPI.

(F) *gata3* ISH in heat-shocked control telencephalon at 3 dpl.

(G) *gata3* ISH in heat-shocked Tg(*hsp:dnfgfr1-gfp*) telencephalon at 3 dpl. Scale bars 20 μm. n = 4 fish for (B), 6 for (C), 6 for (D and E), and 5 for (F and G). Data are represented as mean ± SEM.

(H) Heat-shock and amputation scheme for Tg(*hsp70l:dnfgfr1*) fish. (H′) *gata3* ISH on 2 dpa nontransgenic siblings. (H″) *gata3* ISH on 2 dpa Tg(*hsp70:dnfgfr1*) fish.

(I) Transient and brief heat-shock and amputation scheme for Tg(*hsp70l:dnfgfr1*) fish. (I′) Gata3 and GFP immunostaining on nontransgenic siblings shown for single fin ray. Dashed lines, amputation margin. (I′1–I′3) Individual fluorescence channel for Gata3, DAPI, and merged image. (I″) Gata3 and GFP immunostaining on Tg(*hsp:dnfgfr1-egfp*) animals shown for single fin ray. Dashed lines, amputation margin.

(J) *spry4* expression at 24 hr postfertilization (hpf) embryo.

(K) Blocking Fgf signaling abrogates *spry4* expression.

(L) *gata3* expression at 24 hpf.

(M) Blocking Fgf signaling does not alter the expression of *gata3*. (J′–M′) Dorsal view of animals in (J, K, L, and M), respectively. n = 5 for (H–H″), 3 for (I–I″), and more than 50 embryos for (J–M′). Scale bars 200 μm.

See also Figure S4.

effect might be indirect because of the extended blockage time, and Fgf signaling might act via intermediate signaling pathways to regulate *gata3* expression. To address this issue in the brain, we blocked Fgf signaling transiently by a single heat shock at 2 dpl and analyzed the expression of *gata3* at 3 dpl (Figures S4A–S4A″). We observed that a single heat shock to express dominant negative *fgfr1* reduced but did not abrogate the injury-induced expression of *gata3* (Figures S4A–S4A″). Combined with the data on fin regeneration (Figures 4H–4I″), these results suggest that Fgf signaling has a direct effect on expression of *gata3* after injury in zebrafish tissues and that this interaction represents an injury-dependent mechanism.

We found that blocking *gata3* activity in the ventricular region does not affect wound closure and resolution of blood clotting at the lesion site in the adult zebrafish brain (Figure S4B). Additionally, generalized stress conditions or incision wounding does not induce *gata3* expression (Figure S4C). These results suggest a specific requirement of *gata3* in regeneration programs of zebrafish and that *gata3* expression upon injury correlates with the regenerative ability, which might be controlled by genetic programs that are fundamental to the initiation and maintenance of the regenerative response. A better understanding of these programs might allow us to devise ways to unlock the regenerative potential in humans. Overall, we propose that zebrafish activates molecular programs that may link the nonphysiological conditions of an injury, which are often detrimental for regeneration in mammals, to the initiation of redevelopment of the lost structures, and that these programs are distinct from the ones involved in constitutive stem-cell maintenance and physiology. Specifically, *gata3* may represent an integral component of a molecular program that is used universally for regeneration in zebrafish. Thus, studies on activating injury-associated signaling pathways involving Gata3 in mammalian tissues might be a promising approach in regenerative medicine.

EXPERIMENTAL PROCEDURES

Fish Maintenance and Husbandry

All animal experiments and procedures were carried out in accordance with the recommendations and official guidelines for animal handling and research of the Regierungspräsidium Dresden (permit numbers: AZ 24D-9168.11-1/2008-2, 4, and 14). All surgery was performed under anesthesia, and all efforts were made to minimize suffering. Fish were maintained as described (Brand et al., 2002).

Transgenic Animals

Tg(*huC:GFP*) (Park et al., 2002), Tg(*olig2:GFP*) (Shin et al., 2003), Tg(*her4.1:GFP*) (Yeo et al., 2007), and Tg(*her4.1:mCherry*) (Kroehne et al., 2011) were used as reporters. For blocking Fgf signaling, Tg(*hsp70l:dnfgfr1-GFP*) (Lee et al., 2005) was used. Animals were heat shocked after lesion until 3 days postlesion (dpl) on a daily basis for 1.5 hr in a 37°C water bath.

Stab Lesions, Cell Sorting, and Quantitative Real-Time PCR

Injuries to telencephalon were performed as described (Kroehne et al., 2011) on 6-month-old zebrafish. Total RNA was isolated from (Tg(*her4:GFP*))-sorted cells using Trizol (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed on zebrafish tissues as described (Kizil et al., 2009).

Tissue Preparation, In Situ Hybridization, and Immunohistochemistry

Tissues were prepared as described (Kizil et al., 2009, 2012b). Fish brains and hearts were cryosectioned at 12 μm thickness. *gata3* (NCBI accession number

NM_131211) was isolated from 2 dpf zebrafish total cDNA (Supplemental Experimental Procedures). mRNA probes were generated using in vitro transcription kit (Roche, Indianapolis, IN, USA). In situ hybridization on sections was performed using VSi In Situ Robot (Intavis, Koeln, Germany). Immunohistochemistry was performed as described (Grandel et al., 2006; Kizil et al., 2009; Ganz et al., 2010) (Supplemental Experimental Procedures). Incisions and hematoxylin and eosin staining was performed as described (Kizil et al., 2009).

Injections of Vivo Morpholinos

Cerebroventricular microinjection was performed as described (Kizil and Brand, 2011). Splice- (this paper) and translation-blocking (Yang et al., 2010) and control vivo morpholino oligonucleotides were used (Supplemental Experimental Procedures).

Transient Overexpression by Plasmid Transduction in the Adult Zebrafish Brain

We suspended 0.25 fmol of desired plasmid in 0.4% Lipofectamine (Invitrogen) and Dulbecco's modified Eagle's medium. Fish were injected as described (Kizil and Brand, 2011). Analysis is performed at 2 days after injection.

Image Acquisition and Processing

Images were taken using a structured illumination microscope (Apotome, Zeiss, Oberkochen, Germany) and a confocal microscope (LSM780, Zeiss). Single-stack images were acquired. Image*JA* v.1.45b, Adobe Photoshop 7.0, and Corel Draw X5 were used to generate figures.

Quantifications and Statistical Analyses

Tissue sections (12 μm) between the olfactory bulb and the caudal telencephalon were used for statistical analyses (8–12 sections). Quantifications from one brain (multiple sections) were analyzed for normal distribution using the NORMDIST function of Excel software (MS Office). All data sets conformed to normal distribution (NORMDIST value < 0.005). Paired t test was used for p value and significance (equal number of data points) to compare two data sets. Quantifications for same time points from different brains or different time points for same type of treatment were analyzed using two-way ANOVA with posttest (alpha: 0.05; for comparisons of more than two data sets; Analysis ToolPak, MS Excel, and GraphPad Prism) and heteroscedastic t test (for comparisons of two data sets with different number of data points). Significance is denoted with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. Bars in graphs represent SEM.

SUPPLEMENTAL INFORMATION

Supplemental information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.10.014.

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